Origina article

Prenatal screening service for fetal RHD genotyping to guide prophylaxis: the two-year experience of the Friuli Venezia Giulia region in Italy

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Background - Fetal *RHD* genotyping of cell-free fetal DNA (cff-DNA) from RhD-negative pregnant women can be used to guide anti-D prophylaxis: the knowledge of fetal RhD type can direct and restrict the use of prenatal anti-D immunoglobulin exclusively to RhD-negative women carrying a RhD-positive fetus. Since November 2019 in the region of Friuli Venezia Giulia (Italy) a prenatal screening service has been offered to RhD-negative women at 22-24 weeks of gestation.

Materials and methods - The cff-DNA is extracted from a simple peripheral maternal blood sample to analyze the fetal *RHD* gene: the results are interpreted as *RHD*-positive fetus, *RHD*-negative fetus, or Inconclusive. The service is shared with all regional hospitals and tests are provided free of charge by the National Health System.

Results - Overall, 142 RhD-negative pregnant women were recruited in nearly 2 years. Fetal *RHD* genotyping was negative in 53 pregnancies and positive in 89 pregnancies. Thus, unnecessary treatment of pregnant women and exposure to a scarce plasma-derived medicinal product was avoided, by the use of a single blood sample, in 37.8% of cases, representing 100% of the RhD-negative women carrying a RhD-negative fetus in our cohort.

Discussion - The first Italian region-wide screening service for fetal *RHD* genotyping has been implemented for 2 years, despite the COVID-19 pandemic, in order to obtain the predicted fetal RhD phenotype before the 28th week of gestation, during which prenatal prophylaxis is usually administered. Giving prenatal anti-D immunoglobulin exclusively to RhD-negative women carrying a RhD-positive fetus reduces the overall use of anti-D immunoglobulin, which is becoming an ever more limited resource. The high sensitivity of the procedure provides evidence that the implementation of a diagnostic test in a reference laboratory guarantees the quality of the results, the concordance of reports and the sustainability of costs, representing an excellent guide to targeted use of prophylaxis.

Keywords: *hemolytic disease of the fetus and newborn, targeting prenatal prophylaxis, non-invasive prenatal diagnosis, fetal RHD genotyping, screening service.*

INTRODUCTION

RhD incompatibility in pregnancy is the major cause of maternal alloimmunization and can result in the significant and potentially fatal consequence of hemolytic disease of the fetus and newborn[1]. It is very well established that the risk of RhD alloimmunization and

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the number of subsequent cases of hemolytic disease of the fetus and newborn are drastically reduced by the use of routine anti-D immunoglobulin prophylaxis: firstly, in the 1960s by the introduction of postnatal prophylaxis reducing the risk from 10-15% to 0.8-1.5% and then by the combination of antenatal and postnatal prophylaxis in the late 1990s which further decreased the risk to 0.18-0.35%[2-5]. There is, however, concern regarding the unnecessary administration of Rh immunoglobulin to pregnant women, as a considerable number of unnecessary doses of anti-D are given to pregnant women with RhD-negative fetuses. In fact, in the Caucasian population 36-38% of RhD-negative women carry a RhD-negative baby[6]. Moreover, Rh immunoglobulin is a blood product with an inherent risk of infectious disease transmission, a possible cause of adverse reactions and there is a worldwide shortage of this product.

The advent of non-invasive prenatal diagnosis using cell-free fetal DNA (cff-DNA) to determine fetal *RHD* status provided the opportunity to restrict the use of anti-D prophylaxis only to those mothers carrying a RhD-positive fetus[7-10]. The D status of the fetus of pregnant women should be determined using cff-DNA from a maternal peripheral blood sample; this technique has a false negative rate of <1%[11], typically due to small quantities of fetal DNA, whereas false positive results are primarily caused by the presence of pseudogenes or variant genes that do not produce D epitopes[12]. During pregnancy, the fetal DNA increases to approximately 2-40%, constituting a mean of 10% of total cff-DNA across gestational ages[13], and it was demonstrated that the best time to draw blood for the detection of fetal DNA in maternal plasma samples while avoiding false negative results could be brought forward to the second or early part of the third trimester of pregnancy.

The first nationwide screening program was introduced in 2010 in Denmark[14] followed in 2011 in the Netherlands, in 2014 in Finland, and also regionally implemented in Sweden, in the UK, in France, and Belgium with very high assay accuracy and sensitivities of 99.9%[15]. In the Netherlands, in 2 years of the screening experience, 37.1% women avoided unnecessary recommendation of anti-D treatment, which was equivalent to 97.3% of the RhD-negative women carrying a RhD-negative fetus[16].

During 2017 a mandatory validation protocol for fetal *RHD* genotyping of cff-DNA was implemented in the region of Friuli Venezia Giulia in northern Italy, with the aim of introducing the test for targeted antenatal anti-D immunoglobulin prophylaxis in Italy as well[17]. The region of Friuli Venezia Giulia has a resident population of nearly 1,200,000 people, with 7,900 births/year (2020 data). Taking into account the number of Rh-negative pregnant women (15% in a Caucasian population), the screening was predicted to involve about 1,185 women/year.

Here we report the data from the first 2 years of the screening service for fetal *RHD* genotyping at gestational week (gw) 22-24 to predict the fetal RhD type. This region-wide service was implemented during the coronavirus disease 2019 (COVID-19) pandemic, so causing a recruitment slowdown and a drastic reduction of samples collected compared to the expected ones; nevertheless, the challenges, in terms of organization, compliance and diagnostic accuracy, were met successfully.

MATERIALS AND METHODS

Patients' recruitment and sample collection

According to national recommendations for the prevention and treatment of hemolytic disease of the fetus and newborn, provided by the Italian Society for Immunohematology and Transfusion Medicine (SIMTI) in 2014, all pregnant women undergo blood group typing (ABO, RhD) and antibody screening before the 12th gw. Since November 2019 fetal *RHD* genotyping has been offered to all non-immunized RhD-negative women at 22-24 gw in an antenatal screening program in the region of Friuli Venezia Giulia.

The service is shared with all regional hospitals and tests are provided free of charge by the National Health System. After obtaining the patients' informed consent, two 6 mL samples of peripheral blood are collected into ethylenediaminetetraacetic acid (EDTA)-containing separator tubes simultaneously with other routine blood samples. Identification and clinical data are organized in separate databases, respecting a distinction based on the gestational week. Within 48 hours after sampling, plasma is separated from each blood tube, initially by centrifugation at 3,200 rpm for 10 minutes at room temperature, and subsequently by centrifugation at 10,000 rpm for 10 minutes at 4°C. Each supernatant is portioned into 1 mL aliquots, stored in fresh polypropylene cryogenic vials at –20°C before the following analyses, performed

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at the Immunohematology Reference Laboratory of the Department of Transfusion Medicine, Azienda Sanitaria Universitaria Friuli Centrale, Italy. All the samples tested for this study were actual clinical samples.

Cell-free fetal DNA extraction

DNA was extracted from 2 mL of a plasma sample using an automated procedure with a QIAsymphony® DSP Circulating DNA Kit (QIAGEN, Hilden, Germany). A final elution volume of 60 µL was obtained, thus allowing a real-time quantitative polymerase chain reaction (PCR) analysis to be carried out in duplicate for each extracted sample. The cff-DNA extracted was immediately used for PCR analysis or stored at –20°C until further testing.

Fetal *RHD* genotyping

The presence of fetal *RHD* sequences in cff-DNA was determined using the Free DNA Fetal Kit® RhD (Institute de Biotechnologies Jaques Boy, Reims, France). The kit included: *RHD*-positive and *RHD*-negative plasma samples as positive and negative controls; an exogenous DNA (maize DNA) as an extraction and amplification control, and four sets of primers and hydrolysis probes specific for *RHD* exons 5, 7, 10 and for maize exon *IVR2*. Real-time quantitative PCR analysis was performed using a CFX96 Real-Time System apparatus (Bio-Rad, Hercules, CA, USA): 5 µL of DNA template were tested in a final reaction volume of 20 µL, thus obtaining the plasma-equivalent per PCR of 500 µL. All samples were run in duplicate. Results were considered acceptable only if no amplification curve was observed for the negative and blank controls, the cycle threshold (Ct) values for exons 5, 7 and 10 for the positive controls were below 39 cycles and the exogenous DNA (maize) was correctly amplified (Ct value below 37 cycles) during the assay. If these pre-defined conditions were all fulfilled simultaneously, it was possible to proceed with the sample evaluation. Fetuses were classified as *RHD*-positive or -negative according to the following interpretation of the results: the absence of amplification (Ct null) for the three exons identified a negative *RHD* sample; otherwise, Ct values between 35 and 41 cycles for two or three exons identified samples as *RHD*-positive. Results in between were considered inconclusive. A default baseline was used.

Validation approach

The diagnostic method was validated before routine implementation by performing a total of 423 tests[17]. The diagnostic accuracy was evaluated by correlating the predicted fetal *RHD* genotyping and observed RhD phenotype status of the neonate at birth, which represents the standard reference.

RESULTS

The flow chart followed for all pregnant women to prevent hemolytic disease of the fetus and newborn is shown in **Figure 1**. The timing of the antibody screening and fetal

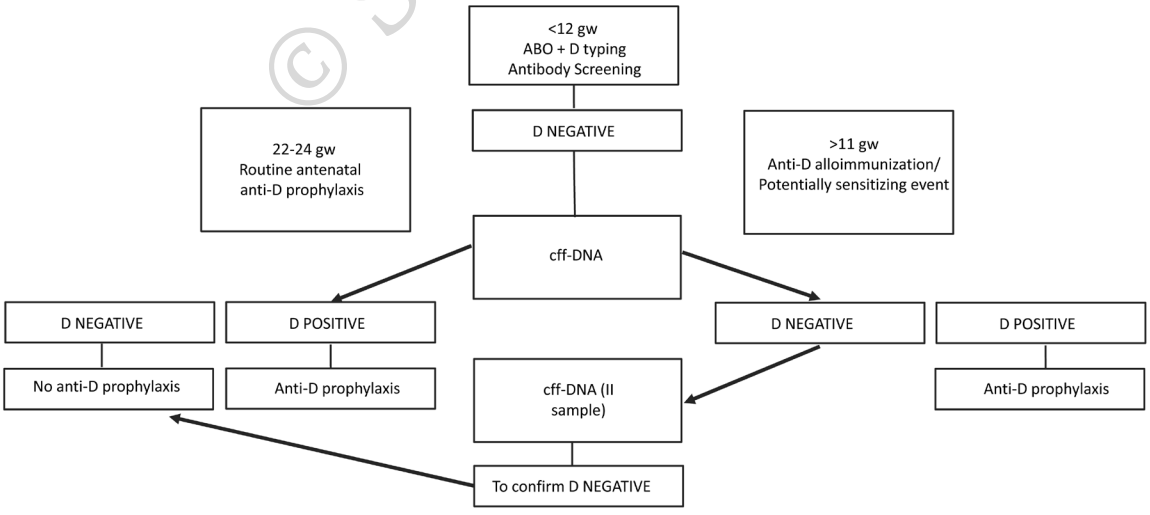

Figure 1 - Flow chart for the prevention of hemolytic disease of the fetus and the newborn in pregnancy showing the with timing of diagnostic investigations
gw: gestational week; cff: cell-free fetal.

RHD genotyping to guide targeted use of Rh prophylaxis in our clinical setting are indicated.

In particular, at the first antenatal visit (<12 gw), all pregnant women undergo ABO and RhD typing and red cell antibody screening; subsequently, fetal *RHD* screening and a newly performed red cell antibody screen are offered at about 22-24 weeks of pregnancy to all non-RhD-immunized D-negative pregnant women. If a D-positive fetus is predicted, antenatal anti-D prophylaxis (1,500 IU, 300 µg of anti-D immunoglobulin) is administered as a single dose in week 28 of pregnancy and another dose of 1500 IU anti-D is administered after birth (within 48-72 h after delivery) if antibody screening is negative, according to national guidelines. The red cell antibody screening is also repeated in week 28 for all pregnant women.

From November 2019 to November 2021, 142 RhD-negative pregnant women were recruited at different gestational ages (between 10 and 31 gw, median 24) and cff-DNA was extracted from maternal plasma to analyze the fetal *RHD* gene (exons 5, 7 and 10). Nearly 80% of cases were collected in the second trimester (14-27 gw), with 30% of these between 22-24 gw, as shown in **Figure 2**.

The fetal *RHD* genotype was determined in 140 out of the 142 cases (98.6%). In two cases inconclusive results were obtained but they were considered *RHD*-positive for the purpose of administration of prophylaxis. In the first inconclusive case only exon 10 was amplified and the presence of a Rh variant was suspected as the mother was previously genotyped as *RHD*01N.05* (also known as hybrid allele *RHD-RHCE (3-7)-RHD*); in the second case the Ct values for exons 5, 7 and 10 were below 35, so maternal genome contamination was suspected as the mother's genotype was known to be *RHD*01W.5*. At birth serology on cord blood samples of these two cases showed a negative Rh type in the first baby and a positive Rh type in the second one. **Figure 3** shows the amplification plots and related Ct values for the two inconclusive cases and for a *RHD* positive sample.

The diagnostic accuracy of fetal *RHD* genotyping on cff-DNA was evaluated by comparing the real-time

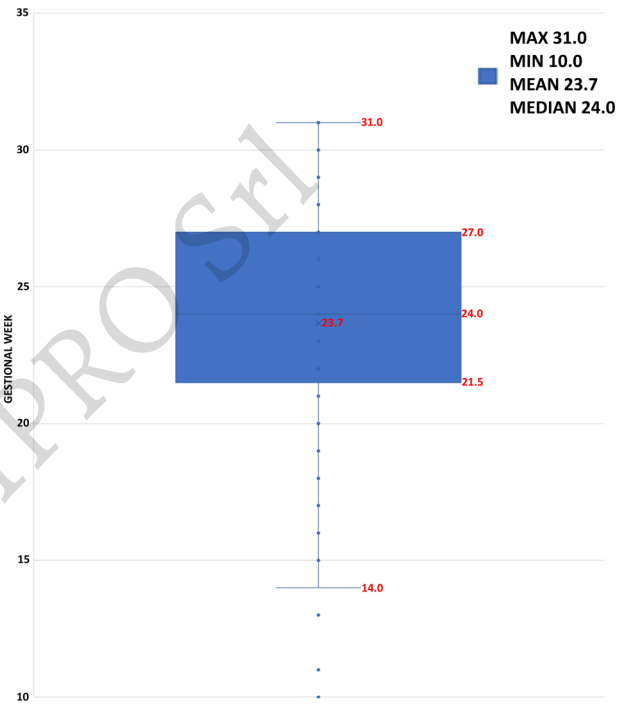

Figure 2 - Box plot of gestation weeks at which blood was collected from patients scheduled for fetal *RHD* genotyping

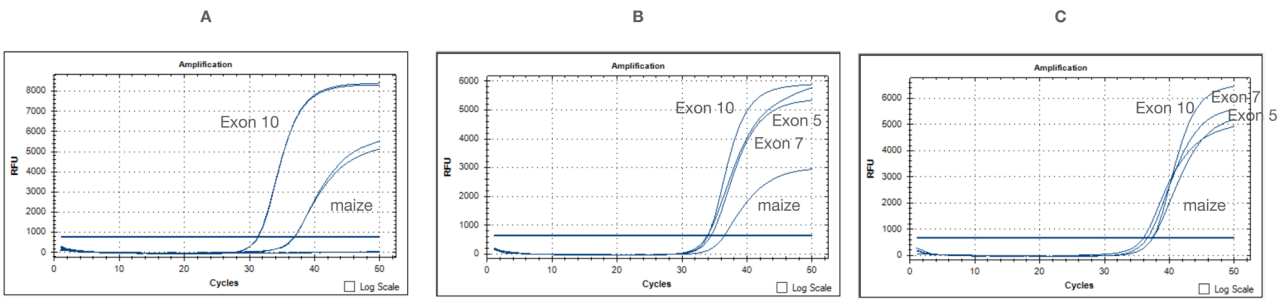

Figure 3 - Amplification plots and related cycle threshold values (Ct) for the two inconclusive cases and for a *RHD* positive sample

Plot A represents the inconclusive case with only exon 10 amplified (Ct = 31.35); plot B represents the inconclusive case with the Ct values for exons 5, 7 and 10 below 35 (Ct=33.89, 34.43, and 34.01, respectively); plot C represents a *RHD*-positive case (Ct=37.45, 36.63, and 37.41).

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quantitative PCR results with postnatal RhD phenotype serologically determined on cord blood samples: 116 out of 142 serological tests were obtained at the time of the study, representing 81.7% of all analyzed samples, also including samples from two twin pregnancies, with both couples of twins confirmed as RhD-negative by serology as predicted by fetal *RHD* typing. Furthermore, among the fetal *RHD*-positive results, a sample with RhD-discrepant serological typing was found at birth: *RHD* genotyping was repeated on a sample from the neonate and a *RHD*01.W2* variant was found, thus demonstrating the high sensitivity of the fetal *RHD* screening test. Likewise, the excellent reproducibility of the test was confirmed by the use of replicates of two samples tested first in early gestation (<11 gw) with *RHD*-negative results and then retested, as required by our protocol, around 22-24 weeks of gestation: both the predicted fetal *RHD* genotypes were confirmed and later were then concordant with the observed RhD phenotype of the neonates at birth. No requests were made for new samples because of failed tests.

Table I shows the performance characteristics of the assay with particular attention to the diagnostic accuracy by comparing its results with postnatal cord blood serology results.

DISCUSSION

RhD-negative pregnant women carrying a RhD-positive fetus are at risk of developing anti-D antibodies during or after pregnancy. Alloimmunization is preventable by using antenatal and postnatal anti-D prophylaxis in clinical routine, thus considerably reducing the frequency of hemolytic disease of the fetus and newborn. As the fetal RhD status can be determined with high sensitivity and accuracy from the mother's peripheral blood, targeted antenatal anti-D-prophylaxis is becoming a new standard procedure in ever more countries, which have introduced non-invasive fetal *RHD* genotyping in a clinical setting of targeted antenatal prophylaxis and management of RhD-sensitized women[18-21]. After validation of a diagnostic method in terms of analytical sensitivity, lower limit of detection, analytical specificity, assay precision and diagnostic accuracy[17,22], we have implemented for 2 years the first Italian region-wide screening service for fetal *RHD* genotyping, despite the COVID-19 pandemic, in order to predict the fetal RhD phenotype before the 28th gw, during which prenatal prophylaxis is usually administered. Besides the mandatory validation steps, however the implementation of any diagnostic test requires adaptation of organizational aspects and establishment of an educational and communication network for the purpose of involving clinicians. After optimizing routine laboratory organization and continuous training of qualified personnel, the first challenge was to bring the new diagnostic test to the attention of medical practitioners and obstetric care providers by sharing scientific results and clinical benefits. The second task was to prepare a simple and effective operational algorithm that is easy for clinicians and users to follow, starting with being able to request the test within the services provided by the regional health system. With regard to the first point, after an encouraging start, the program implementation slowed down, with the number of recruited samples below expected, because of the COVID-19 pandemic. However, we have now observed an improvement in compliance, contextually with a slight improvement in the emergency situation, with the possibility of sharing information and reaching as many users as possible in our region. As far as concerns the operational algorithm, the greatest constraint was that of choosing the best gestational week in which to perform the test. In fact, for mass throughput screening of all D-negative pregnant women cff-DNA testing for *RHD* is sufficiently accurate from 11 weeks gestation with false-negative rates of 0.1-0.3%[18,22-24]; however, it is well documented that the choice of collecting the sample between 18 and 24 weeks of gestation is a consequence of the recommendation to use a single sample, for reasons of cost-

Table I- *Performance characteristics of the prenatal RHD screening test*

Results from antenatal *RHD* screening	
Total number of non-invasive prenatal tests	142
Screens with available postnatal RhD result	116
True positive results	67
True negative results	47
False-negative results	0
False-positive results	1*
Inconclusive results	2
Sensitivity (%)	100%
Specificity (%)	97.9%
Accuracy (%)	99.1%

Specificity, sensitivity and accuracy are based on all data, including inconclusive results. *One sample categorized as inconclusive due to positivity of exon 10 alone, was later considered a false positive, when the cord blood sample was found to be D-negative.

effectiveness and timely reporting of results to clinicians before the 28th gw. We chose a restricted blood sampling period between 22 and 24 gw, recruiting women from a multi-ethnic population. All our negative *RHD* genotyping findings were confirmed at birth, suggesting that under these operating conditions, *RHD* genotyping on a single sample would be sufficient and that *RHD* genotyping on cff-DNA from RhD-negative women can be used to guide targeted antenatal prophylaxis for the prevention of RhD immunization. Furthermore we showed that our assay can be used on subjects from different ethnic backgrounds. We observed that the gestational ages of the samples received deviated from the scheduled 22-24 weeks, and this will be a point to discuss with clinicians in an effort to standardize the screening program. The knowledge of fetal RhD type can direct and restrict the use of prenatal anti-D immunoglobulin exclusively to RhD-negative women carrying a RhD-positive fetus (60% of individuals of European descent). In our 2-year experience, 37.8% of the women were correctly recommended not to receive antenatal prophylaxis. This approach optimizes the use of anti-D prophylaxis and unnecessary exposure to plasma-derived medicinal products[7,18,25-27].

CONCLUSIONS

To our knowledge, the present study is the first prenatal screening service for fetal *RHD* genotyping to guide prophylaxis in Italy.

Overall, 142 RhD-negative pregnant women were recruited in a period of nearly 2 years. The fetal *RHD* genotype was determined in 140 (98.6%) cases and was negative in 37.8% of cases and positive in the other 62.2% of cases.

We demonstrated that it is simple to predict fetal RhD phenotype in all RhD-negative pregnant women (previously typed before the 12th gw) before the 28th gw, during which antenatal prophylaxis is usually administered in Italy. The antenatal screening program was effectively implemented, with the collection, storage and automatic extraction of DNA from maternal plasma to analyze the fetal *RHD* gene.

The results confirm that fetal *RHD* antenatal testing is highly reliable and that the centralization of the test in a single laboratory guarantees the quality of the results and the concordance of reports. It represents an excellent guide for targeted use of Rh immunoglobulin in pregnancy and customer satisfaction has been excellent The data were collected during the COVID-19 outbreak and we hope that this screening service will be enhanced in the future, being confirmed as a useful tool for healthcare organizations and patients' outcome in Friuli Venezia Giulia and other Italian regions.

AUTHORSHIP CONTRIBUTIONS

DL designed the study. SM and CD collected the data. SM and DL wrote the manuscript. GB supervised the study. All Authors critically reviewed the manuscript.

The Authors declare no conflicts of interest.

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